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12b. DISTRIBUTION CODE**13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**

Estrogen (E2) is required for the development of breast cancer, but there are few animal models to study its mechanism of action in vivo. We hypothesize that the timing of estrogen receptor α (ER α) action is crucial in normal mammary gland development and tumorigenesis. Therefore, a transgenic mouse model with a regulatable ER α is being generated. In vitro, a mutant ER α (525L) has a severely attenuated response to endogenous estradiol (E2) but a wild-type (WT) response to diethylstilbestrol (DES). E2 binding was decreased in 525L (Kd 12.5nM) compared with WT (Kd 0.1nM), but DES binding was comparable (Kd 0.04 and 0.03nM respectively). Gene targeting was used to insert 525L into the WT ER gene. Screening for the construct in 350 ES cell clones is underway. The mutant 525L ER α discrimination in transcriptional response to DES and E2 appears to result from differences in ligand binding affinity. The resultant transgenic mouse will enable us to study how the timing of ER activation modulates normal mammary maturation and the development of mammary cancers.

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INTRODUCTION

Estrogen and its receptor are required for normal breast development and are important in the treatment and prevention of breast cancer. However, there are few suitable animal models to study the role of ER activation in the breast development and cancer. We developed a mutant ER having a severely attenuated response to endogenous estradiol while demonstrating a wild-type response to a synthetic estrogen agonist *in vitro*. We postulate that the timing and nature of estrogen action is crucial in normal mammary gland development and tumorigenesis. We are proposing a transgenic mouse model with a regulatable ER to test this hypothesis. Our specific aims are to 1) Develop transgenic mice using gene-targeting techniques expressing only the ligand discriminating mutant ER. 2) Define the contribution of ligand induced transcriptional activation of ER in the development of normal mammary gland. 3) Investigate how the timing of ER activation modulates the development of genetically and chemically induced mammary cancers. The mutant ER mice are uniquely suited to study the role of ligand independent ER activation *in vivo*, and will also be useful in studying reproductive tract development and behavior.

BODY

Task 1. Transgenic mice expressing only a mutant ER with ligand discrimination will be developed. (months 1 - 12)

a) Mouse ER genomic DNA will be cloned. A targeting construct (8 to 10 kb) will be generated.

Construct (Work performed at University of Chicago): The 15 kb construct used in

subsequent experiments is composed of mouse genomic sequence from ER α (exons 8-9 and external downstream sequence), cloned into the pZero vector (Invitrogen) at the multiple cloning site, XhoI (5' end of insert) and HindIII (3' end of insert). A Neomycin resistance cassette,

consisting of the PGK promoter, neo open reading frame and poly A tail sequence, is found approximately 1.2 kb downstream of exon 9 in reverse orientation at a BamHI site found in the genomic DNA. For removal with Cre-recombinase, the Neo cassette was flanked by LoxP sites (Figure 1). This construct was provided by our collaborators Deborah Swope and Ken Korach¹. Approximate restriction enzyme sites and distances between sites were determined by sequencing and restriction enzyme/DNA gel analysis.

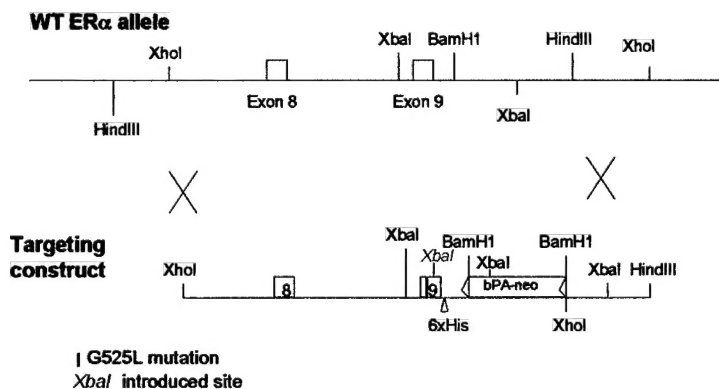


Figure 1

Mutagenesis (Work performed at University of Chicago): Mutagenesis was performed by PCR using Qiagen's site directed mutagenesis kit. Three rounds of mutagenesis were performed following the kit's instructions. Briefly, primers were designed using MacVector to 1) alter the coding sequence at codon 525 to convert glycine (G) to leucine (L). 2) Create a novel XbaI site in exon 9 while maintaining the amino acid sequence. 3) Remove a nucleotide insertion event (4G→3G) near the end of exon 9 that was discovered upon sequencing. Each round of mutagenesis was performed as directed, with the PCR extension time expanded to accommodate 15 kb.

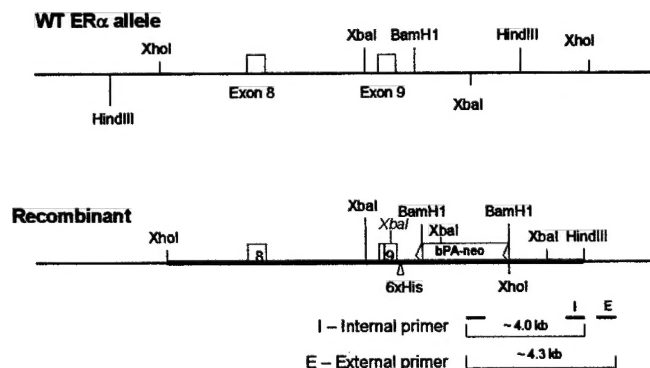
Ligand binding characterization of 525L mutation (Work performed at Medical College of Wisconsin): We hypothesized that the ligand discrimination of 525L is a result of differences in binding affinity between E2 and DES present in 525L and not in WT ER α . Charcoal assays were used to determine saturation binding characteristics. The 525L mutant receptor had a K_d of 0.03nM with DES, compared with a K_d of 12.5nM with E2. In contrast, the WT receptor had a K_d of 0.04 with DES, and a K_d of 0.12nM with E2.

b) Mice transgenic and homozygous for mutant ER will be generated (Work performed at Medical College of Wisconsin).

1) **Homologous recombination in embryonic stem cells.** 2×10^8 embryonic stem cells (D3) were electroporated with 100 microgram of linearized targeting construct. Transfected cells were plated onto 20 dishes containing a confluent monolayer of neomycin-resistant, growth-inactivated (gamma-irradiation) embryonic fibroblasts. 24h after transfection, the growth media was supplemented with G418 (final concentration 200 microgram/ml) and Gancyclovir (final concentration 1 micromolar). After 9 days, approximately 350 resistant colonies were isolated and individually expanded for preparation of frozen stocks and for preparation of genomic DNA.

2) **PCR Strategy for detection of homologous recombination in ES cell clones (Work performed at University of Chicago):** For identifying positive ES clones that have replaced the endogenous estrogen receptor sequence with the altered construct, a PCR strategy was designed. Primers (external) were designed (MacVector) to bind to a region near the end of the neo open reading frame and to a region just outside of the construct (sequence from BLAST mtrace database) (Figure 2). If the construct is incorporated into the mouse genome at the appropriate

Figure 2



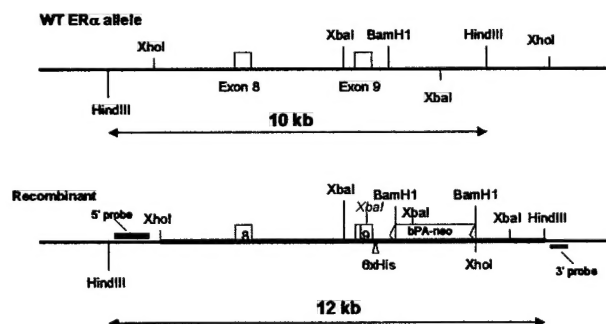
location, a 4.3 kb band will be generated. In addition, a primer was designed to a region to the end of the construct (Figure 2). This internal primer (4.0 kb band) will allow us to confirm the validity of PCR as well as identify PCR conditions before ES cells are electroporated. (*The following work performed at Medical College of Wisconsin*) The PCR strategy was tested using genomic DNA from a positive ES clone (using the same targeting construct but with a different mutation) identified by our collaborator, Debra Swope and Ken Korach. However, we were unable to detect any PCR product with either the internal or external primers from the ES cell clone, though the internal primer set amplified the construct DNA without difficulty. We tried varying conditions, *Taq* enzymes, alternate primer sets, increasing the amount of DNA template, all to no avail. We suspect our failure in the ES cell DNA template is due to 1) the small amount of starting template and resultant small amount of product that could not be detected by ethidium bromide staining, and 2) inefficiency of amplification due to the length of PCR product (~4000 bp) required to confirm homologous recombination. At this point, we are reluctant to use labeled probes for detection of PCR product due to potentially increasing the chance of identifying false positive samples from contamination. Therefore, we have changed to a Southern hybridization strategy for detection of homologous recombination in ES cells.

3) *Southern hybridization for detection of homologous recombination of ES cell clones (Work performed at Medical College of Wisconsin):* 50 ul of genomic DNA from

each clone was digested with HindIII and incubated overnight at 37°C. The DNA was precipitated, washed, dried and dissolved in loading buffer. It was then loaded onto a 0.7% agarose gel and run overnight. The gel was denatured and washed with 10x SSC. Overnight transfer in 10x SSC was followed by washing the blot in 2X SSC, drying, and UV crosslinking. The

probes (provided by Dr. Swope, figure 3) were prepared using random primer reactions with Klenow enzyme and 32P-dATP. Sephadex G50 columns were used to separate free nucleotides from the probe, and specific activity was calculated. Prehybridization for 30 minutes was followed by hybridization with the probe (1 x 10⁷ cpm of probe /ml of hybridization solution) overnight at 68°C. Serial washes at 65°C at 2X SSC (twice) and 0.2X SSC (twice) were followed by overnight exposure to film at -80°C and processing.

Figure 3



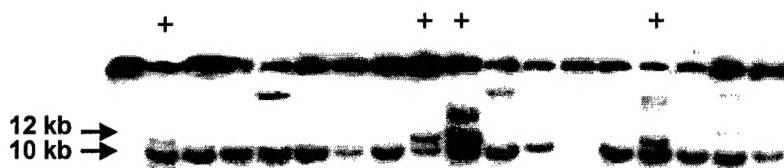


Figure 4

To date, 9 of 200 ES cell clones hybridized with the 5' probe has the insert. The wild type band is 10 kb, the recombinant is 12 kb due to addition of the 2 kb neo cassette (Figure 4).

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Demonstration that ligand discrimination of 525L is a result of differences in binding affinities between E2 and DES.
- 2) Creation of targeting construct (2 versions).
- 3) Electroporation of targeting construct into ES cells with resultant 350 clones, screening underway.

REPORTABLE OUTCOMES:

Abstracts:

- 1) Sugg SL, Li J, Huang JJ, Radek J, Brace J, Swope DL, Korach KS, Greene GL. Design of a transgenic mouse with a regulatable estrogen receptor alpha. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orlando 2002.
- 2) Sugg SL, Li J, Radek J, Shilyansky J, Greene GL. Differences in binding affinity causes mutant estrogen receptor alpha ligand discrimination. Submitted to Society of Surgical Oncology 2003.

CONCLUSIONS

Over the first year of this award, we have made good progress toward creating the transgenic mouse model with a regulatable ER that was proposed for this project. We anticipate having transgenic animals within the next 6 months. We will then be able to characterize the phenotype, followed by experiments to determine how the timing of ER action affects mammary gland maturation and modulates mammary tumorigenesis and latency in models of genetically and chemically induced mammary cancers. With the widespread clinical use of ER ligands, detailed understanding of ER action is increasingly important. This model will allow us to manipulate ER transcriptional activity *in-vivo* during different developmental stages, and will add significantly to current knowledge on ligand-activated and ligand-independent ER action. Results from our proposal on how the timing of ER activation modulates normal mammary maturation and the development of mammary cancers will likely lead to determining susceptible periods for treatment in the prevention of breast cancer in humans.

REFERENCE

1. Swope DL, Castranio T, Harrell JC, Mishina Y, Korach KS. AF-2 Knock-In Mutation of Estrogen Receptor α : Cre-*loxP* Excision of a PGK-neo Cassette From the 3' UTR. *Genesis* 32:99-101, 2002.